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(54) **Surface second harmonic and sumfrequency generation immuno and enzyme assays**

(57) This invention relates to the use of nonlinear optical methods of surface second-harmonic generation

and sum-frequency generation to detect immuno and enzyme reactions and nucleotide hybridisation.

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## Description

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention.

The detection and measurement of a large number of compounds within complex biological and biochemical material is a basic requirement of clinical and forensic laboratories and is increasingly important in environmental monitoring and the biotechnology industry. This invention relates to the use of nonlinear optical methods of surface second-harmonic and sum-frequency generation to detect and quantify antibody-antigen interactions, polynucleotide hybridisation and enzyme-substrate complexes.

#### 2. Description of the Prior Art.

The basic principal that underlies nearly all quantitative immunoassays is the addition of a reagent to a test sample which results in the formation of a complex of the reagent and a specific component within the sample. The concentration of the reagent which remains unbound compared to that which is bound provides a measure of the specific component. In many (heterogeneous) tests it is necessary to separate the unbound from bound reagent. Usually this is done by some physical process which is often time consuming, expensive and wasteful of manpower and materials. In other tests where secondary procedures are employed to visualise the formation of complexes, there is often a long incubation period before the reaction can be detected. Other problems with conventional immunoassays include the expense, short useful half-life and toxicity of the reagents. For example, one of the most sensitive and widely used assay techniques is radioimmunoassay. However, this technique suffers from both the health hazard posed by the radioactive reagent and waste products, as well as a useful lifetime which is limited by the half-life of the radioactive label.

Two strategies are commonly employed in non-radioactive detection (1) direct methods where enzymes or fluorophores are incorporated into the probe (2) indirect where probes are modified by ligands (biotins/haptens) and detected after by conjugates of ligand-specific proteins with lanthanide or enzymes. Optical detection methods include absorbance and fluorescence measurements.

Methods which do not require separation of free and bound reagent are known and employ the unique optical features presented at interfaces between media. One method that exploits this feature is surface plasmon resonance; this has been commercialized to detect biospecific reactions (BIAcore: Pharmacia®, Sweden). Surface light-bending and evanescent waveguide immunosensor techniques have also been described (U.S. Pat. 4880 752). There are no reports or patents describing

the use of nonlinear optical signals for bioassays.

### SUMMARY OF THE INVENTION

The present invention consists of a method for immunoassay, polynucleotide hybridization and enzyme assay that exploits changes in the properties of the medium in the interfacial region resulting from the binding reaction that can be detected by nonlinear optical techniques.

The essential features of the invention are:

① The utilisation of a nonlinear optical process to directly quantitate an interaction between biological molecules (antibodies, antigens, polynucleotides, enzymes, enzyme substrates and analogues).

② The detection is surface specific so that the assay can be performed in the presence of unreacted components/species that do not interact with the surface layer. This eliminates the need to separate the bound and unbound components.

### DESCRIPTION OF THE PREFERRED EMBODIMENTS

Fig. 1 shows that second-order optical processes such as sum- and difference-frequency generation are forbidden in media with inversion symmetry but such symmetry will necessarily be broken at an interface, where the nonlinear processes are thus allowed. Second-order optical processes originate from the field and structural discontinuity at the interface. Therefore alterations in the surface e.g. by the formation of a surface molecular layer 2 leads to a measurable change in the detected surface second-harmonic signal 3. The signals are also strongly dependent upon the angle  $\theta$  of excitation-emission vector with respect to the surface and degree of order of molecular orientation. Therefore, if the orientation or surface nonlinear coefficient of a surface layer is altered e.g. by the formation of a complex between the surface molecules and a second molecule the characteristics of the second-harmonic signal (e.g. polarisation, magnitude and direction) will also be altered.

The surface nonlinear susceptibility will exhibit a resonant enhancement when the output frequency of the laser  $f$  or  $2f$  is close to a molecular excitation of a surface species (antigen, antibody, polynucleotide, enzyme, enzyme substrate or substrate analogue). This may be achieved by use of a suitable laser or a tuneable laser or alternatively by covalent or other attachment of a reporter molecule, which possess a molecular excitation close to  $f$  or  $2f$ , to the antigen, antibody, polynucleotide, enzyme, enzyme substrate or substrate analogue thereby producing a condition of resonance enhancement.

A simple extension of the present invention occurs

when the light incident upon the interface contains two frequencies  $f_1$  and  $f_2$  (e.g. when two lasers are employed) then  $f_1 = f_2$  may no longer be true. In this case the output is no longer at  $2f$  but at  $f_1 + f_2$  such a case constitutes sum-frequency generation. This is still a second-order nonlinear optical effect which is surface specific and shares the other properties of surface second-harmonic, as described above. In addition, if the antigen, antibody, polynucleotide, enzyme, enzyme substrate or substrate analogue or a ligand attached to any of these above mentioned molecules has a distinct electronic or vibrational transition, surface sum-frequency generation can be used to selectively detect and quantify the presence the molecule possessing the particular transition at the surface or changes in the molecule, when located at the surface, which affect the said transition. This may be achieved by ensuring that at least one of the output frequencies of the source corresponds to the target transition. The same effect can be achieved by using a tuneable source. Surface sum-frequency generation, according to an application of the invention, can be used as a basis of a non-separation surface-based bioassays as described in this specification.

Enhancement of the nonlinear optical signals can be achieved by:

1. Attachment of ligands to any of the interacting species that serve to increase the structural and/or electric field discontinuity at the surface.
2. Attachment of a charged particle to any of the interacting species that change the surface electric field on complex formation.
3. Attachment of ligands which possess magnetic properties to any of the interacting species which alter the magnetic field at the interface on complex formation.
4. Attachment of ligands with a large hyperpolarizability to any of the interacting species whose orientation is altered by complex formation.
5. Attachment of charged or magnetic species or groups with a large hyperpolarizability to the surface whose properties are altered by complex formation so as to modulate the nonlinear optical signal.

Refinement of the nonlinear optical signal (increase in signal to noise ratio) can be achieved as described in Fig 2. The signal 5 has the exact temporal profile as the laser excitation pulse 4 and can be easily time gated whereas fluorescence life-time 6 depends on local environment.

A number of distinct advantages of a surface sensing scheme based on surface second-harmonic generation over existing optical assay schemes are obvious.

1. The surface second-harmonic signal is up-shifted in frequency. It is therefore well removed from potential optical noise sources such as fluorescence and phosphorescence of the target species and background luminescence associated with components in biological material, which are all down-shifted in frequency. Interference from the excitation source is also minimised.

2. The large up-shift in the frequency makes filtering the signal against unwanted background light straightforward.

3. Accurate time-gating of the signal, eg. using a boxcar, can reduce spurious signals by direct comparison with the excitation pulse thus improving the signal to noise ratio of the assay.

4. Being a laser-induced process it is coherent and highly directional.

5. Modulation of the nonlinear optical signal by application of external electric or magnetic fields, especially when charged or magnetic materials are located in the surface, can be used to discriminate the signal.

6. The sensing scheme offers potential reductions in time, cost (per test) and skilled manpower requirements over assay systems presently in use.

in the invention antibodies, antigens, polynucleotides or enzymes are attached to the sensor surface. In one embodiment of the invention multiple tests may be performed where the surface comprises spatially-separated surface domains to which different antibodies and/or antigens and/or polynucleotides and/or enzymes and/or enzyme substrates and/or enzyme inhibitors are attached. The surface is then brought into contact with a solution which may contain the complementary species ie antigen, antibody, polynucleotide or enzyme inhibitor. Formation of a complex between the complementary species will result in a modification of the surface nonlinear optical properties. Measurement of the magnitude, angular dependence or any other parameter dependent on changes of nonlinear optical properties such as surface second-harmonic generation can be used to determine the amount of complex formation at the surface.

A schematic diagram for detection of nonlinear optical signals is shown in Fig. 3. The beam of a Q-switched Nd:YAG laser 7 is directed through a polariser 8 onto the sample 9 mounted on a calibrated rotation stage 10. The nonlinear optical signal passes through a neutral density filter 11, a 1062nm blocking filter 12 and a 532nm band pass interference filter 13 and is detected by a photomultiplier tube 14. The detector response is fed, together with that recorded from a frequency dou-

bled beam produced by a frequency doubler 15, into a boxcar 16 and the output dumped to a computer 17 for processing and display.

Different formats for interrogating surfaces are illustrated in Figs. 4-6. Fig. 4 shows a waveguide 18 in a total internal reflection format with the biomolecular layer 19 located at the surface. A direct surface probe format is shown in Fig. 5 which is suitable for transparent surfaces consisting of glasses or preferably polymer plastics in which there is greater scope for mutually orienting molecules on the surface. The incident light beam 20, after interacting with the biomolecular layer 21, is transmitted 22 and reflected 23 and its intensity recorded using detectors 24. Signal selectivity can be enhanced using both transmitted and reflected beams by exploiting its angular dependence. The format shown in Fig. 6 is suitable for metal surfaces and other nontransparent surfaces since the incident light beam 25 can interact with the biomolecular layer and the reflected beam 26 can be detected; the transmitted beam 27 can be detected if the material is transparent. Measurements can be performed after removal of the sensor surface from contact with the solution containing unbound reagents (dip-stick format) or while in contact with the solution.

Glass, polymer and metal surfaces are found to exhibit strong surface second-harmonic signals which are modulated in proportion to the amount of randomly-oriented biomolecules absorbed onto the surface. A comparison of a conventional ELISA assay of bovine serum albumin (BSA) using anti-BSA antibody/HRP-linked anti-IgG antiserum with the surface second-harmonic generation method is shown in Fig. 7. Fig. 7A is the result obtained for dilution of antibody in the ELISA assay performed in a standard Nunc® plastic assay tray. Fig. 7B is the nonlinear optical signal intensity obtained at 45° to the incident beam recorded from the underside of the same wells shown in Fig. 7A. Similar results were obtained from samples containing only BSA and anti-BSA antibody. Fig. 7C is a comparison of the two methods.

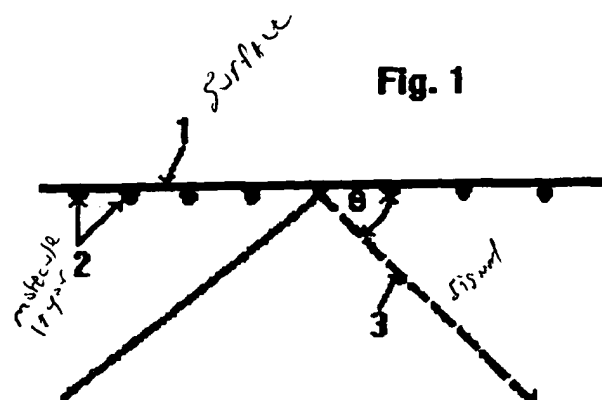
The invention can be used for all types of immunoassay including those involving immunoglobulins, specific receptor proteins, hormones, drugs and like compounds and all enzymes for which an inhibitor is known. Enzyme assays in one aspect of the invention are configured by the use of specific inhibitors which bind to the catalytic site according to well characterised association constants (competitive inhibitors). Inhibitors are known for most enzymes that are closely related structurally to the substrate but are not reactive. These inhibitors compete with the substrate for binding to the catalytic site of the enzyme. In one aspect of the invention the change in the surface second harmonic signal produced by binding of the substrate to the surface bound enzyme is used to determine the substrate concentration. In another aspect of the invention inhibitor may be modified or labeled to enhance the field or structural discontinuity at the surface produced by the binding

of the inhibitor to the enzyme. Such a technique could be used for continuous monitoring/assays.

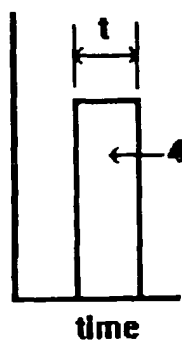
Polynucleotide hybridisation can be detected by changes in nonlinear optical signals which occur when polynucleotide in solution hybridizes to a complementary polynucleotide attached to the surface.

## Claims

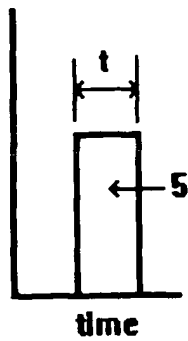
1. A non-separation method for assay of antibodies or antigens or polynucleotides or enzymes or enzyme substrates or enzyme inhibitors in a soluble phase in contact with a surface to which specific antibodies or antigens or polynucleotides or enzymes or enzyme substrates or enzyme inhibitors are localized and whose interaction causes a change in nonlinear optical properties that can be detected and quantitated by surface second-harmonic generation or sum-frequency generation.
2. A method for continuous assay of a substrate present in a product stream or body fluid or environmental domain of an enzyme localized at a surface monitored by changes in nonlinear optical properties of the surface resulting from interaction of the enzyme with the substrate or interaction of the enzyme with a competitive inhibitor of the enzyme reaction in the presence of the substrate.
3. A method as described in 1 or 2 where the surface comprises a glass, polymer or metal layer with antigens or antibodies or polynucleotides or enzymes or enzyme substrates or enzyme inhibitors attached or adsorbed and where complex formation alters the nonlinear optical properties of the surface layer.
4. A method as claimed in 1 where polynucleotide hybridisation is detected by binding of an antibody specific for double-stranded polynucleotide.
5. A method as described in 1 or 2 where the change in the surface nonlinear optical properties on complex formation is enhanced by attaching a ligand to any of the interacting species that will increase a structural and/or electric field discontinuity at the surface on complex formation.
6. A method described in 1 or 2 in which the nonlinear optical properties of the surface layer is modulated by application of external electrical fields.
7. A method as described in 1 or 2 where complex formation is performed so as to result in a mutual orientation of the interacting species at the surface.



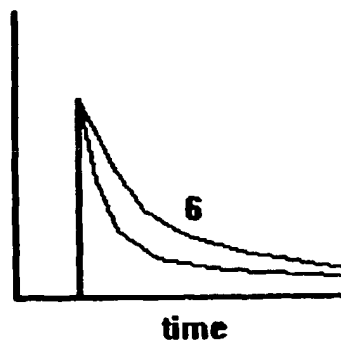
**Laser Pulse**



**SSH signal**



**Fluorescence**



**Fig. 2**

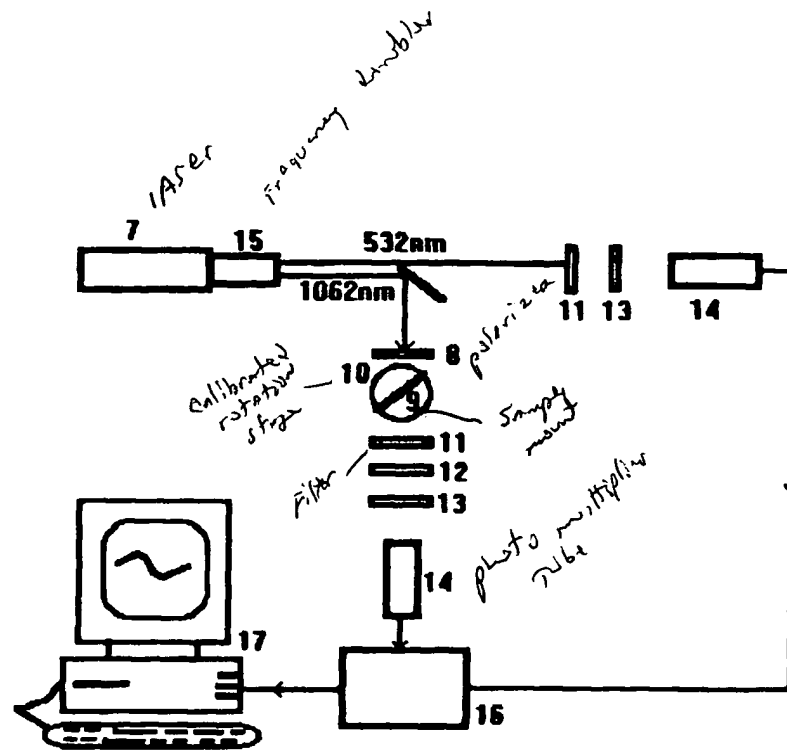
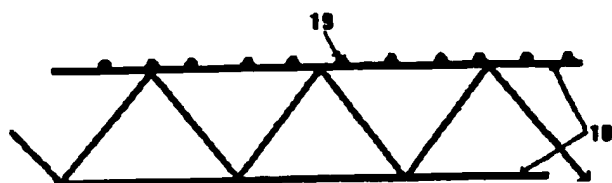
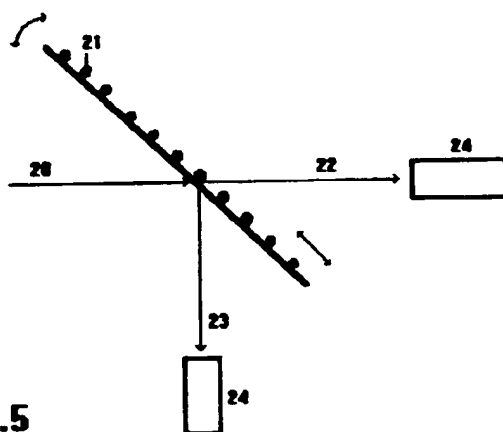


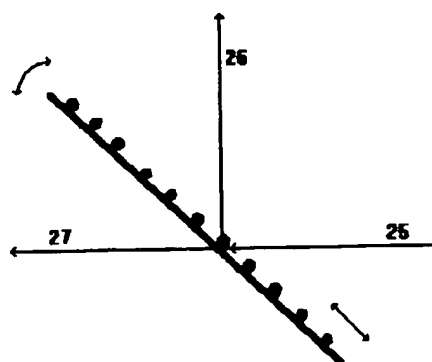
Fig. 3



**Fig. 4**



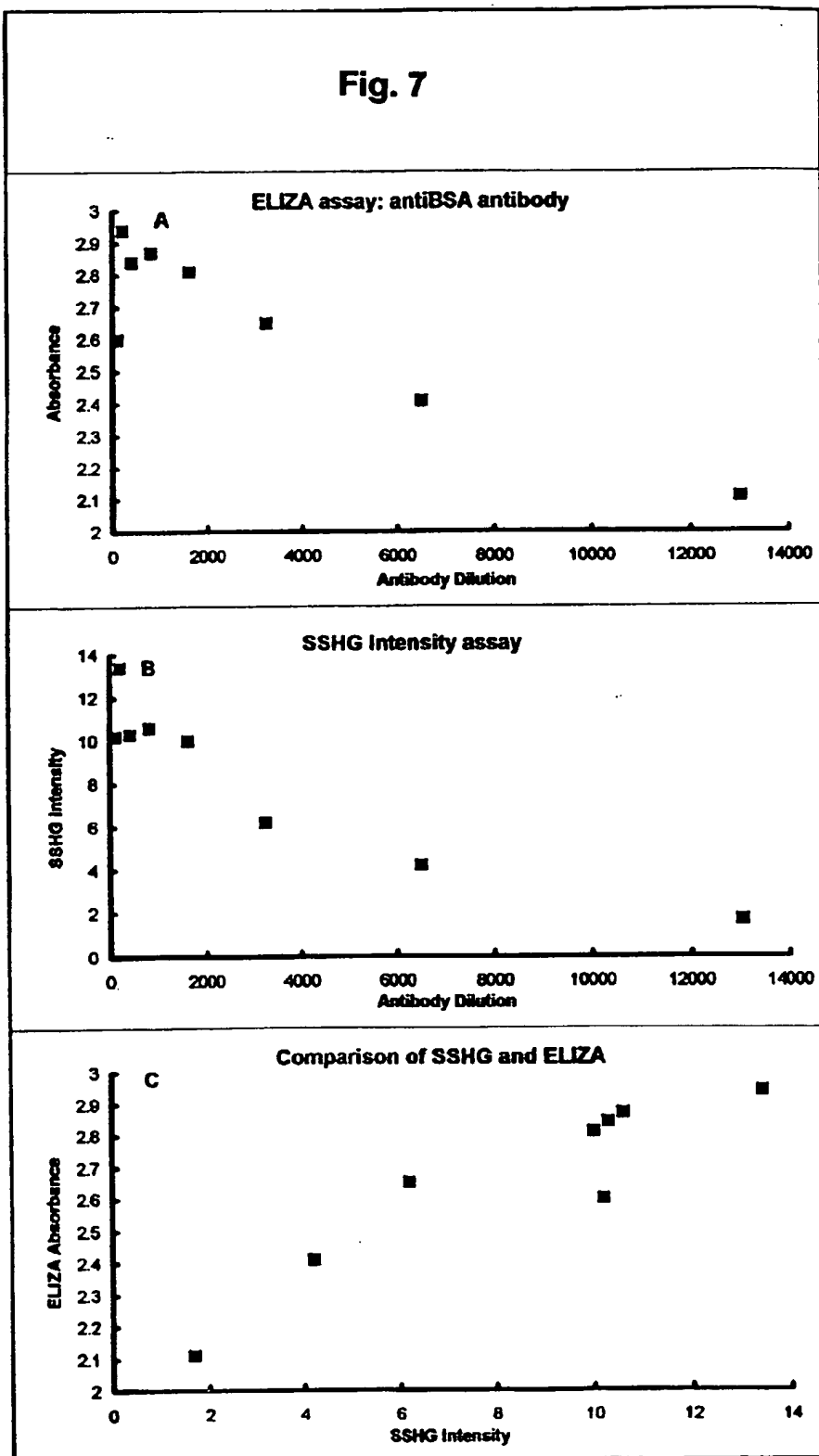
**Fig. 5**



**Fig. 6**



**Fig. 7**





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## EUROPEAN SEARCH REPORT

Application Number  
EP 96 30 2858

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,A	EP-A-0 175 585 (CORNING GLASS WORKS) 26 March 1986 * the whole document *	1,2	G01N33/543 C12Q1/68
A	DATABASE WPI Section Ch, Week 9006 Derwent Publications Ltd., London, GB; Class B04, AN 90-039569 XP002009683 & JP-A-01 314 949 (CANON KK) , 20 December 1989 * abstract *	1,2	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
			G01N C12Q
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 30 July 1996	Examiner Moreno, C
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			

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